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Usefulness of 'Cyprinodon variegatus' and 'Fundulus grandis' in Carcinogenicity Testing Advantages and Special Problems

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Usefulness of *Cyprinodon variegatus* and *Fundulus grandis* in Carcinogenicity Testing: Advantages and Special Problems[1, 2]

Lee A. Courtney *and* John A. Couch[3]

ABSTRACT—*Cyprinodon variegatus* and *Fundulus grandis*, two species of cyprinodontid fishes studied extensively and used in toxicological and biological investigations, are compared as laboratory test animals. Their ecology, general biology, and suitability for various types of experimentation are examined. A laboratory system for exposure of these species at critical life stages (embryos, fry, juveniles) to suspect carcinogens is described. A discussion of the use, findings, and potential of these species in oncological studies and carcinogen assays is presented, particularly in regard to responses to 3 known or suspect carcinogens (trifluralin, benzidine, and aflatoxin). Finally, advantages and disadvantages or special problems in using the species as carcinogen test animals are reviewed. — Natl Cancer Inst Monogr 65: 83–96, 1984.

For years, the cyprinodontid fishes have been a source of abundant adaptable organisms for research. *Cyprinodon variegatus* Lacépède 1803, commonly known as the sheepshead minnow, an important species in the estuarine ecosystem, is widely distributed, easily collected, and well suited for laboratory culture and research. The general biology and ecology of *C. variegatus* have been studied extensively since 1900, and many references to systematics and collection date back well into the 1800s. During the past 10 to 15 years, the sheepshead minnow has been used routinely by scientists for testing toxicity of environmental pollutants and contaminants. Refined culture techniques have enabled researchers to hold and breed this adaptable species in flow-through, recirculating, and static systems year round at coastal facilities or in locations far from any salt water source. *C. variegatus* has proved valuable in acute and chronic toxicity tests, reproduction studies, tests for pathological and physiological responses, and, more recently, carcinogenicity.

Cyprinodontid fishes of the genus *Fundulus* have also provided researchers with excellent test organisms. *F. similis* Baird and Girard, *F. confluentus* Goode and Bean, *F. heteroclitus* Linnaeus, and *F. grandis* Baird and Girard have been used for various studies ranging from developmental investigations to pesticide testing to assay of lunar material collected during the Apollo Program (*1*). Extensive studies have been made of the life history, general biology, and ecology of many *Fundulus* species. For example, *F. heteroclitus* has been used widely in developmental and embryological studies for over 50 years (*2*) and much of what we know concerning fish embryology was learned with *Fundulus*.

Culture techniques have also been developed for *Fundulus* that allow the production of large numbers of fish for testing or commercial enterprise. The species of particular interest to us in the South Atlantic and Gulf Coast region is *F. grandis*, the Gulf killifish, a close relative of *F. heteroclitus*. Like *C. variegatus*, this species provides an available and easily cultured test organism. However, some significant developmental and physical differences do occur between *C. variegatus* and *F. grandis*. The latter species exhibits an extended embryological development, a longer maturation period, a larger size, and it offers a varied experimental application.

We present here a concise review and comparison of the general biology and ecology of *C. variegatus* and *F. grandis*. In addition, the suitability of each species as a laboratory test animal will be discussed, including culture and holding methods. A brief overview of the sensitivity of *F. grandis* and *C. variegatus* in toxicological testing will follow. Finally, we shall consider the use, findings, and potential of these species in oncological studies and carcinogen assays.

TAXONOMY AND SYSTEMATICS

The killifishes (order Cyprinodontiformes) are found throughout the world. The order was organized into 8 subfamilies under the family Cyprinodontidae by Myers (*3*); however, Rosen (*4*) raised 1 subfamily to familial rank and created a 2-family structure for the order. Taxonomic classification (to subfamily), according to Rosen's 2-family system, and general distribution of the Cyprinodontiformes, are shown in table 1. *F. grandis* and *C. variegatus* are both members of this order.

C. variegatus has occupied a stable taxonomic position since its original description by Lacépède in 1803 (*5*). However, *F. grandis* and several other *Fundulus* species have been the objects of some controversy regarding taxonomic position and relationships (*6–8*).

ABBREVIATIONS: ppt = parts per thousand; H & E = hematoxylin and eosin; LC50 = 50% mortality when the lethal concentration is administered.

[1] Presented at the Symposium on the Use of Small Fish Species in Carcinogenicity Testing, National Institutes of Health, Bethesda, Maryland, December 8–10, 1981.

[2] Supported by the Environmental Research Laboratory of the United States Environmental Protection Agency, Gulf Breeze, Florida 32561.

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TABLE 1.—*Taxonomic classification of the order Cyprinodontiformes to subfamily with general geographic distribution*

Family	Geographic area
Cyprinodontidae[a]	
Subfamily	
Cyprinodontinae	Mediterranean, Persian Gulf, Red Sea, Turkey, northwestern Indian Ocean, and portions of southern North America and northern Central America
Fundulinae	Spain, southern France, Turkey, portions of southern North America, Central America, and parts of the Caribbean
Lamprichthyinae[b]	Lake Tanganyika, East Africa
Orestiatinae[c]	Lake Poopo, Lake Titicaca area of South America
Pantanodontinae	East Africa and Madagascar
Procatopodinae	Africa
Rivulinae	Africa, India, Southeast Asia, East India, South America, Central America, and parts of North America and the Caribbean
Oryziatidae[d]	Japan, The Philippines, Celebes, India, Southeast Asia, and East Indies

[a] All but one genera of killifishes are included.
[b] Only a single genus is included: *Lamprichthys*.
[c] Only a single genus is included: *Orestias*.
[d] Only a single genus is included: *Oryzias*.

Similarities exist between certain members within the genus; for example, *F. grandis* Baird and Girard, *F. heteroclitus* Linnaeus, and *F. grandissimus* Hubbs represent a closely related group. Other trios and pairs of closely related species are also found within the genus (*8*). Whether these represent true species, variants, or subspecies has been debated, and various methods of grouping have been used (*7–9*) with conflicting results, leaving specific distinctions within these groups in question. The significance of these questions is uncertain, and until research shows otherwise, the bearing these minor species or sibling species differences may have on species selection for carcinogen testing may be considered of minor importance.

DISTRIBUTION AND ZOOGEOGRAPHY

Both *C. variegatus* and *F. grandis* are readily available for experimental use. *C. variegatus* ranges southward from Cape Cod, Massachusetts, around the peninsula of Florida, along the Gulf of Mexico and into northern South America [fig. 1; (*10–12*)]. *F. grandis* occupies a more limited territory (fig. 1), occurring from the Matanzas River in northeastern Florida down to the Florida Keys and extending northward again around the Gulf of Mexico and down into Mexico (*13, 14*). Interestingly, *F. heteroclitus*, previously noted as a close taxonomic relative of *F. grandis*, has a range extending northward on the east coast from the northern range limit of *F. grandis* to Anticosti Island in the Gulf of St. Lawrence (*13, 14*). Their distributions thereby provide 2 closely related *Fundulus* species and *C. variegatus* as available test animals along the entire eastern and Gulf coasts of the United States and portions of Central and South America.

The habitats occupied by these species are overlapping, and seine collections within adjoining ranges occasionally result in the simultaneous capture of both. *C. variegatus* prefers shallow brackish areas of pools (small, sandy-bottomed sites in bays, coves, bayous, creeks, tidal cuts, or channels) that tend to be drained or left isolated during low tides (*15–18*). Kilby (*15*) observed that fish select areas where little or no submerged aquatics are present, locations offering access to adjacent marshes for food and shelter from the shallows of open bays and large bodies of water. In addition, habitat preference is influenced by fish size. Only adult or subadult fish are collected in water courses; fish of all sizes are taken from pools, coves, and marshes (*15*).

The *F. grandis* inhabits similar habitats in the estuarine system. Kilby (*15*) stated "species which are characteristic of the water-courses include *F. g. grandis* and *F. similis* when taken with *C. v. variegatus* . . . ." He further states that "*F. g. grandis* occurs most abundantly in outer pools, water-courses, and, to a lesser extent, open waters. . . ." Data gathered by Christmas (*19*) and McIlwain (*20*), and field collections conducted by staff at the Gulf Breeze, Florida, Environmental Protection Agency Laboratory (unpublished observations) support these findings.

Both species are euryhaline. *C. variegatus* has been collected in waters ranging from 0 (*15*) to 142.2 ppt (*21*).

FIGURE 1.—Geographic distribution of *C. variegatus* and *F. grandis*.

Simpson and Gunter (*21*) indicated that high salinity is not a typical or preferred situation but occurs when fish are trapped in ponds where evaporation results in increased salinity. Normal salinity ranges for *C. variegatus* are from 0 to 35.6 ppt, with 20 ppt or less preferred (*15, 22*). *F. grandis* favors salinities of 2.0 to 22.5 ppt (*15, 19*), but, like *C. variegatus,* sometimes occurs at salinity extremes of 0.05 to 76.1 ppt (*21, 23*). An extensive review of salinity tolerances for *F. grandis, F. heteroclitus,* and other *Fundulus* species was done by Griffith (*24*).

C. variegatus and *F. grandis* are also eurythermal. Collections and investigations of *C. variegatus* by Warlen (*17*), Forman (*22*), Copeland (*25*), and Westman and Compton (*26*) indicate a temperature tolerance of 2° to 39° C. Christmas (*19*) reported gathering *F. grandis* at temperatures ranging from 5.0 to 34.9° C. The tolerance of salinity and temperature extremes exhibited by both species indicates that *C. variegatus* and *F. grandis,* hardy species in nature, would be adaptable as test animals in the laboratory.

Moreover, a point of further interest is the transportability of these species, especially that of *C. variegatus.* Embryos, fry, juveniles, or adults can all be transported several hundred kilometers in plastic bags if provided periodic oxygenation. This enables researchers at inland laboratories or locations outside the species' normal ranges to utilize them. From 1977 to 1981, several thousand *C. variegatus* of various life stages were transported from the Environmental Protection Agency Laboratory in Gulf Breeze to an inland university with excellent results.

MORPHOLOGY AND LIFE CYCLE

Sexual dimorphism in adult *C. variegatus* is marked by differences in coloration. In general, the body of males is olivaceous and marked with vertical, dark stripes along its sides. During breeding, the anterior dorsal area from the head to above the pectoral fins turns a deep, iridescent steel blue. The most obvious male coloring characteristic, even in nonbreeding males, is a jet-black bar at the tip of the caudal fin. The sex of young males and fish which, through captivity and manipulation have lost their breeding colors, is easily distinguished by this mark. Females are far less colorful and can be distinguished by a dark spot near the posterior base of the dorsal fin.

Sexual dimorphism in *F. grandis* is also striking. Sex determination is easiest during breeding; at this time, males are recognized by pearly white spots located on the sides and fins. Generally, the body is dark green with highlights of yellow and orange on fins and belly. Females are generally plain and have an olive and silvery body with faint silvery vertical bars. No white spots are found on the body or fins.

The life cycles of *C. variegatus* and *F. grandis* are depicted in figure 2, with approximately comparable developmental stages and average developmental times. Considerable work has been done on the embryology of *C. variegatus* (*27*) and *F. heteroclitus* (*28*). Our observations seem to indicate that the life cycle of *F. grandis* is similar to that of *F. heteroclitus.* Published work on the latter type can serve as an excellent guide in the study of either *Fundulus* species.

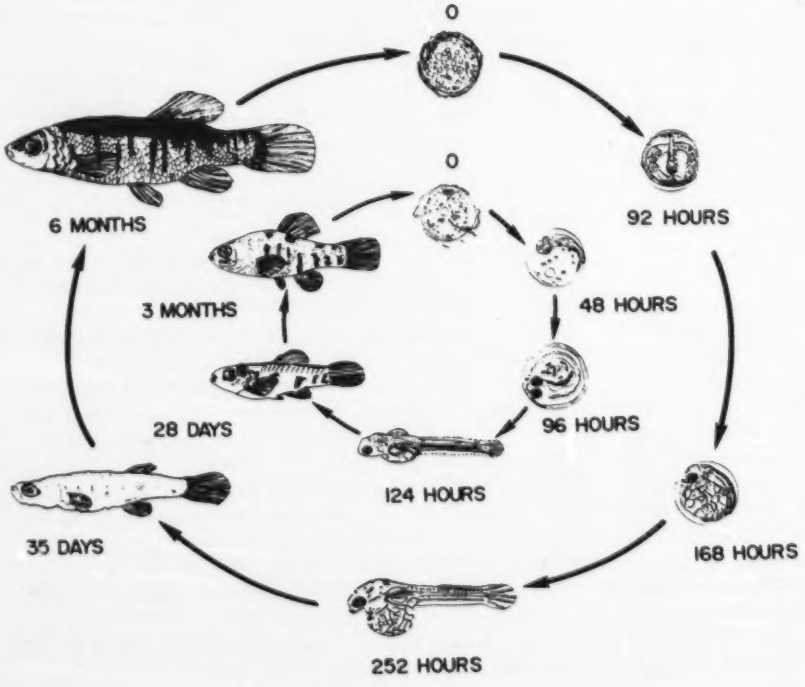


FIGURE 2.—Life cycles of *C. variegatus* (*inner circle*) and *F. grandis* (*outer circle*). Approximately comparable life stages are depicted with average developmental times. Stages are representative of those critical to carcinogen test regimens.

The rapid development of *C. variegatus* makes it an excellent test organism. Hatching, dependent on incubation temperature, occurs in approximately 5 to 15 days, usually 5 to 7 days, unless the temperature drops below 25° C. Generation time under laboratory conditions has been reported at about 3 months (*29*).

F. grandis develops more slowly than *C. variegatus*. The reported developmental period to hatching is 14 to 28 days and depends on the incubation temperature (*30, 31*); we observed similar periods at our laboratory. Tatum et al. (*31*) reported that growth to approximately 56 mm occurred within 52 days at large "grow-out" ponds. Observations on growth of the progeny of feral *F. grandis* spawned in our laboratory have shown reduced growth in comparison with rates reported in large pond operations. Likely causes of this decreased growth are 1) restricted housing space in the glass aquaria used, and 2) a dietary consideration which will be discussed later. These progeny, healthy in appearance and sexually differentiated, have been raised in small 60-liter aquaria for over 20 months but have failed to spawn.

Both *C. variegatus* and *F. grandis* are morphologically typical teleosts. Major organs and tissues in these species generally do not depart in their location or gross form from comparable features in most typical teleosts. In our experiences with *C. variegatus*, only a few exceptions have been noted, the most notable is the possible absence of Stannius corpuscles. Though many sectioned specimens have been examined, we have been unable to identify histologically these structures in anatomic regions where reported in other species, or in any other region of the fish. However, we may have either overlooked these tissues or they are uniquely formed and located in *C. variegatus*. The normal liver histology of this species is variable between specimens and depends on several factors, such as nutritional history and season. Hepatocytes vary in degree of vacuolation caused by removal of glycogen and fat during routine slide preparation. Livers of well-fed specimens (fig. 3) show a great degree of vacuolation (e.g., glycogen and lipid reserve), whereas those of starved specimens show little or no vacuolation (fig. 4). Acini of exocrine pancreas surrounding branches of the hepatic portal vein (fig. 4) and hepatic bile ducts are occasionally found in liver sections.

HISTOLOGICAL PROCESSING

Whole specimens, from zygotes to small adults, can be processed histologically with little difficulty. Figure 5 shows a parasagittal section of a 96-hour *C. variegatus* embryo. Developing organs and organ systems can be studied easily when serial sections are made. Liver differentiation has already occurred at this stage of development, i.e., 96 hours at 26° C (fig. 6); note the structure of hepatocytes in comparison with adult liver sections. Larger specimens (fig. 7) require decalcification before dehydration and embedding. Whole specimens up to 35 mm standard length can be processed and then cut on a longitudinal horizontal plane before embedding. Both halves of the fish are then embedded in the same paraffin block with the cut face on the sectioning plane. Using this method, a researcher can study all organs and major skeletal features with a few selected serial sections from various planes.

At present, the detailed histology of *F. grandis* is under investigation but generally appears similar to that of other teleosts. Embryos and young fish can be processed and studied by the methods described for *Cyprinodon*.

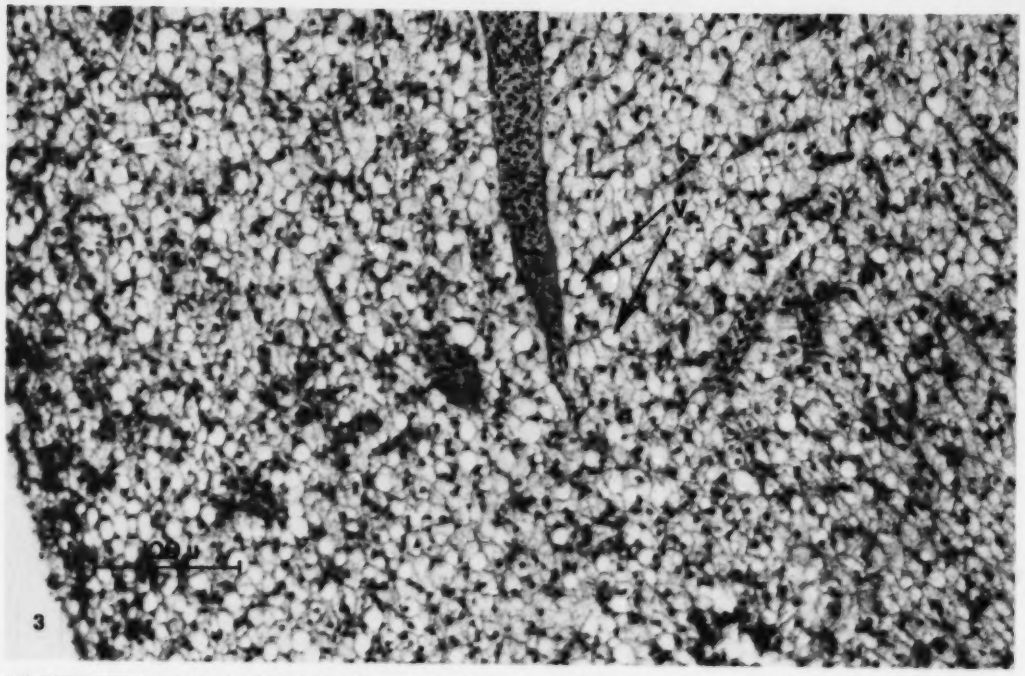
CULTURE

C. variegatus is an easily handled and maintained laboratory research animal. Culture techniques have been developed with either closed systems, under-substrate filter aquaria, or flow-through marine systems (*29, 32*). Adaptations of these laboratory techniques have also been applied successfully to spawning of feral *F. grandis* (unpublished observation), and methods for large-scale commercial production have been developed (*20, 31, 33*) in attempts toward maintenance of the supply of *Fundulus* to the bait industry. Additional culture information for *F. heteroclitus* (e.g., *34, 35*), which is applicable to *F. grandis* as well as other species, is available.

Basically, laboratory culture of *C. variegatus* consists of acclimation (to temperatures between 26° and 30° C and a 12-hour light/dark photoperiod) and conditioning, i.e., feeding ad libitum (*32*). Laboratory breeding can be performed year round once these conditions are met; approximately 2–4 weeks is required for acclimation and preparation of the specimens (*32*).

Once gravid fish are available, 1 of 2 basic methods of culture can be followed, depending upon several factors, including numbers of breeders available, expendability of breeders, and numbers of eggs required for testing. Natural spawning methods (*32*) may be used if parent fish cannot be killed, or if a limited number of eggs are required. With this method, several females are housed with 1 or 2 males in a breeding chamber consisting of a glass enclosure with a large mesh nitex screen bottom (*36*). Natural spawning occurs and the fertilized eggs sink through the mesh bottom. These are collected on a removable screen tray fitted below the enclosure. When 4 to 5 spawning females are introduced to the spawning chamber in the afternoon, between 50 to 120 embryos can be harvested the next morning.

However, if fish are plentiful or expendable, or, most importantly, if large numbers of zygotes are required with critical control over fertilization time, manual spawning methods are indicated (*32*). In this method, acclimated adult females are either dissected or stripped of mature ova which are then placed in a small quantity of seawater. A large harvest of viable ova is ensured by administration of 2 ip injections of human chorionic gonadotropin on consecutive days, beginning 3 days before the fish are killed. The hormone acts as a hydrating agent, inducing maturation of the ova present; this treatment is optional, and the entire manual spawning procedure can be used without injections. Once the mature eggs are collected, the testes of several males are removed and macerated, and milt is introduced into the seawater containing the ova. After approximately 1 hour, the fertilized zygotes can be removed and manipulated as desired.



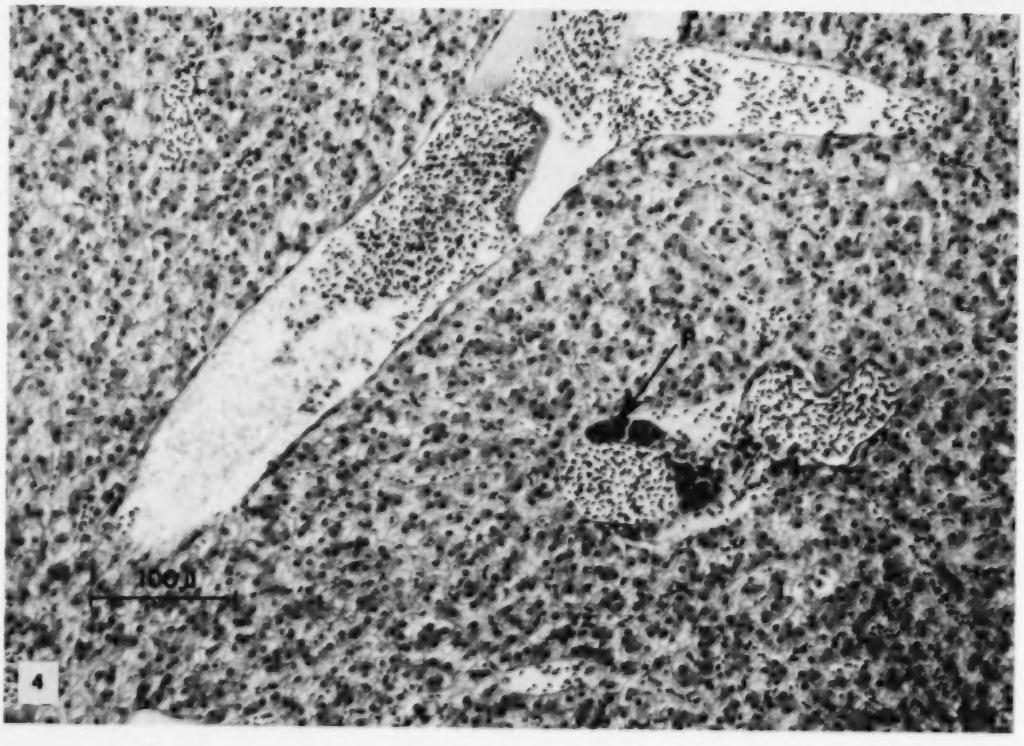
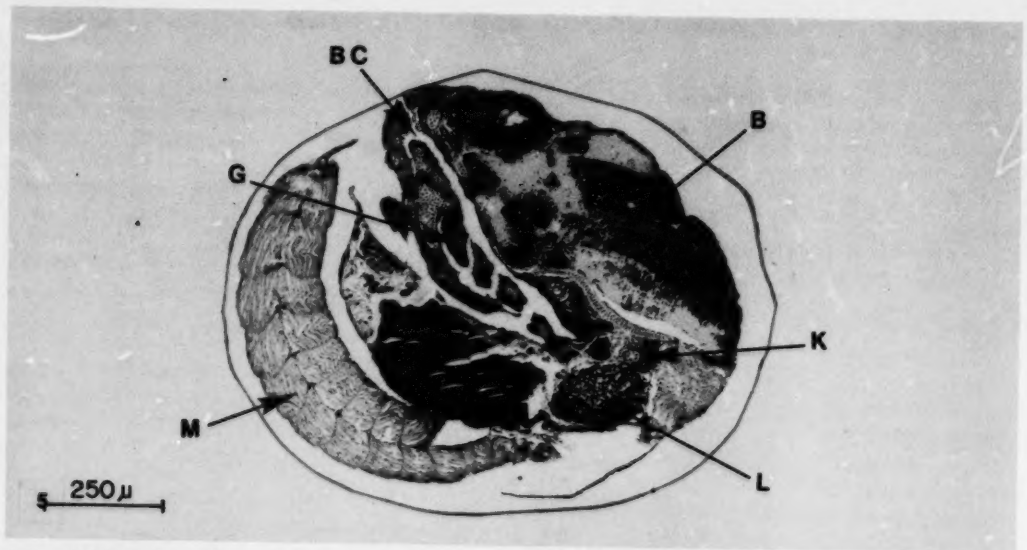


FIGURE 3.—Section of liver from well-fed *C. variegatus*. Hepatocytes show a great degree of vacuolation caused by extraction of stored lipid and glycogen from cells during histological processing. Distribution of lipid reserves is represented by discrete smooth edged vacuoles (V) dispersed throughout the tissue. H & E. × 225

FIGURE 4.—Liver section of a starved individual. Little or no vacuolation is present and hepatocytes appear dense. Acinus of exocrine pancreas (P) is associated with the hepatic portal vein. H & E. × 225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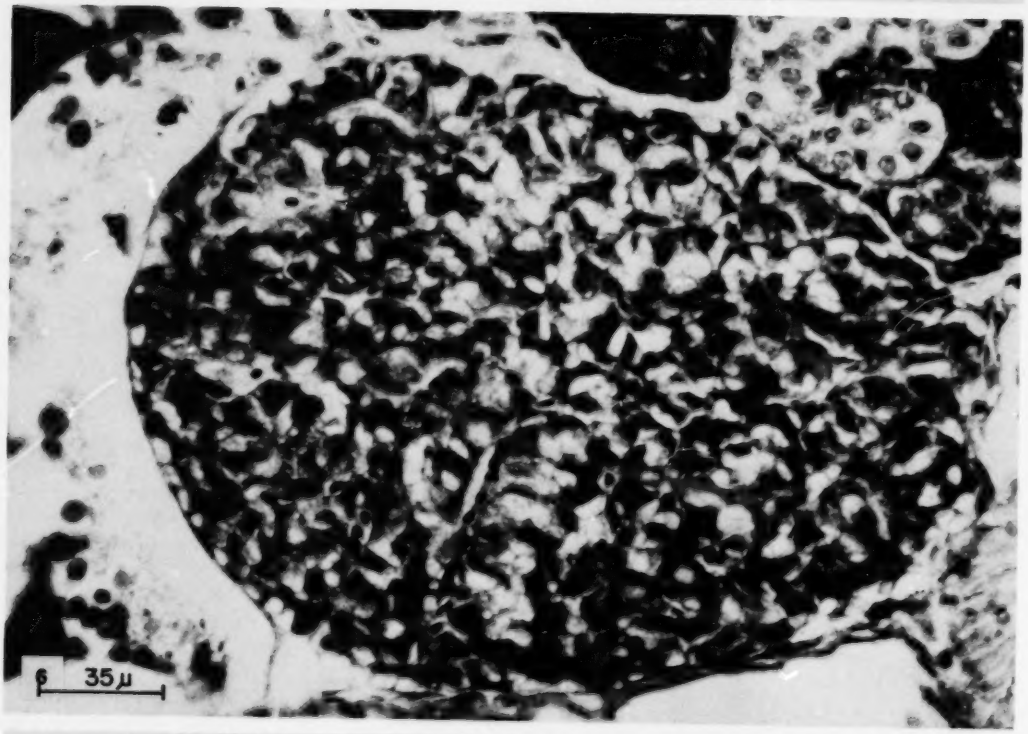
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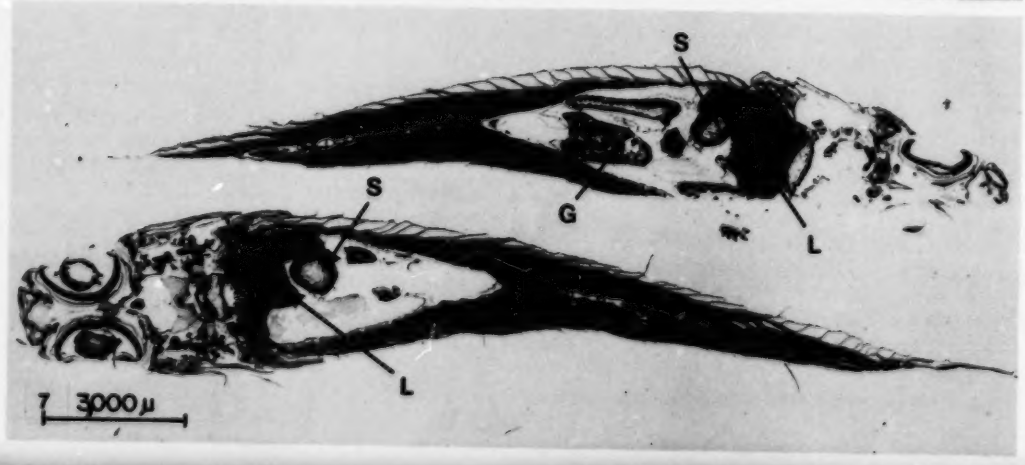
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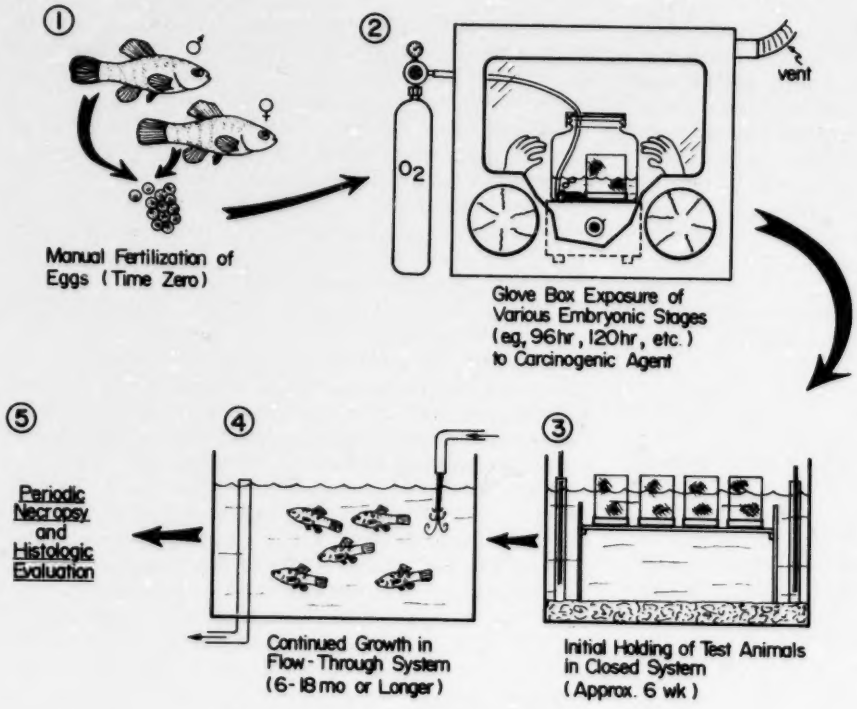
①
Manual Fertilization of Eggs (Time Zero)
②
vent
O2
Glove Box Exposure of Various Embryonic Stages (eg, 96hr, 120hr, etc.) to Carcinogenic Agent
③
Initial Holding of Test Animals in Closed System (Approx. 6 wk)
④
Continued Growth in Flow-Through System (6-18 mo or Longer)
⑤
Periodic Necropsy and Histologic Evaluation

FIGURE 8.—Test scheme for static pulse exposure of selected life stages (e.g., embryos, larvae) to suspect or overt carcinogenic agents. 1) Desired test organisms are obtained by manual methods. 2) Exposure for desired duration of selected stages is performed in closed containers within glove box. Test organisms are held in egg cups within the exposure chamber; a stirring bar evenly distributes and circulates the test agent through the egg cups. Periodic oxygenation of water prevents depletion of dissolved oxygen during exposure. 3) After initial decontamination of test animals in glove box, additional depuration can be accomplished in recirculating systems. 4) This action is followed by extended holding under flow-through conditions, or test animals can be transferred directly to flow-through holding facilities. 5) Multiple observational end points possible during the holding period include physiological, behavioral, biochemical, and histological measurements and assessments.

If stock-rearing is the goal, several hundred embryos can be held in 2,000-ml aerated separatory funnels for a week or more after hatching, then transferred to larger aquaria for grow-out. For testing purposes, 20 to 25 embryos can be held in egg cups, constructed of petri dish bottoms and nitex screen placed in trays designed to provide a fluctuating water level, with excellent results (*37, 38*).

Culture methods described above have been applied successfully to field-collected *F. grandis* (unpublished observation). Although we have held these cultured fish for over 20 months, to date we have had no spawning activity from these specimens.

TEST SYSTEMS

Numerous assay systems have been used in the testing of aquatic organisms. Choice of assay system depends on many factors ranging from type of compound tested to goals of the research project to personal preferences of the researcher.

In our laboratory, we used 2 basic systems in the assay of carcinogens on marine fishes. The first was long-term continuous exposure of various life stages of *C. variegatus* to the herbicide and suspect carcinogen trifluralin according to a system detailed in (*38*). This system, which consists of a water-toxicant delivery system and exposure tanks with various life-stage holding capacity, was for continuous exposure; however, it also could be used for pulse or one-time exposures. We had good results with a simple toxicant mixing chamber; toxicant concentrations ranged between 1 and 5 μg/liter during a 19-month period.

By another assay system (fig. 8), we exposed under static conditions embryos at various developmental stages (critical life stages) to a short single dose of the overt carcinogenic agent aflatoxin B_1. Exposures were conducted in a vented glove box in covered glass containers with the embryos held in egg cups. After exposure, the embryos were rinsed and transferred to uncontaminated egg cups before removal from the glove box, which greatly reduced possible transfer of the carcinogenic agent to the outside

FIGURE 5.—Parasagittal section of a 96-hour embryo of *C. variegatus*. Developing liver (L) can be seen as well as many other organs: brain (B), kidney (K), gill arches (G), buccal cavity (BC), caudal musculature (M). H & E. × 75

FIGURE 6.—Higher magnification of liver from 96-hour embryo of *C. variegatus* in figure 5. H & E. × 570

FIGURE 7.—Longitudinal horizontal sections of adult *C. variegatus*. Specimen was decalcified before processing, and entire fish was cut in the longitudinal horizontal plane immediately before embedding. Both halves of fish were embedded in the same paraffin block with the cut face on the sectioning plane. Therefore, sections pictured represent two planes of the same specimen. All organs and major skeletal features can be studied with this method with selected serial sections from various planes. Sections of liver (L), stomach (S), gonads (G) and other tissues are represented. H & E. × 7.4

environment. After transfer from the exposure cup and several rinses, most of the unabsorbed and unadsorbed compounds were removed; thus the health hazard was reduced. Once exposure and decontamination were completed, embryos were maintained in preconditioned recirculating aquaria through hatching and for several weeks thereafter. At that time, the young fish were moved to larger flow-through holding aquaria. This system, patterned after methods described by Hendricks et al. (*39*) for use with rainbow trout embryos, provided a safe, inexpensive method of one-time exposures of embryos and larval fishes. Exposures with *C. variegatus* were executed with ease and with deaths of fewer than 10% of the fish during the first 6 weeks.

NUTRITION

Nutritional requirements for *C. variegatus* and *F. grandis* differ. Fry of both species thrive on newly hatched brine shrimp (*Artemia* sp.). Young *C. variegatus* should have their diet supplemented with flake food (e.g., BiOrell or Tetramin)[4] and, as their size increases, with frozen adult brine shrimp which has been thoroughly thawed. Adults can be maintained with excellent results on a diet of equal parts flake food and frozen brine shrimp (*40*). We followed a similar feeding regimen with *F. grandis,* and although the fish appeared healthy at sexual differentiation, some growth retardation was noted. Therefore, young adult *F. grandis* should be fed, or, at least a portion of their diet (~50% or more) should be a commercial floating trout feed, such as Purina Trout Chow[4] (*31*), possibly supplemented with vitamins (*20*). This diet has shown promise in studies with pond-reared fish reported by McIlwain (*20*) and Tatum (*31*). All dietary ingredients should be analyzed for possible contaminants, such as pesticide residues or heavy metals. Commercially prepared trout food should be screened for aflatoxin contamination, due to the use of cottonseed oil and other ingredients in its preparation.

ROLES IN TOXICOLOGICAL TESTING

Toxicological testing with *C. variegatus,* begun in the last 30 years, has been extensive, and acute toxicity studies proliferated in the last two decades. Chronic toxicity studies were pioneered in 1974 at the Gulf Breeze Laboratory. Numerous compounds, including polynuclear aromatic hydrocarbons, insecticides, herbicides, and others, have been tested against this species, which displayed its responsiveness and sensitivity as a test organism. A detailed review of the toxicological uses and results of *C. variegatus* would be impractical and unnecessary here, but a few examples illustrate the responsiveness of the species.

Acute toxicity tests have shown that the sheepshead minnow's sensitivity to toxicants is comparable to that of other fishes from similar ecosystems. Schimmel and Hansen (*29*) reported comparisons of 48-hour effective concentrations for the teleosts *C. variegatus, F. similis, Leiostomus xanthurus,* and *Mugil cephalus* exposed to endrin, dieldrin, and dichlorodiphenyltrichloroethane, which indicated comparable susceptibility. Ninety-six-hour exposures of *C. variegatus* to endrin (LC50 = 0.34–0.37 μg/liter) showed comparable susceptibility with data generated on 10 species of estuarine fish [*C. variegatus* was more sensitive than 6 of these species (*36*)]. Furthermore, comparative toxicological responses were observed for *C. variegatus* and *Lagodon rhomboides* in tests with hepatochlor (*41*); these species, however, were less sensitive than was *L. xanthurus.* In other instances, the sensitivity of the sheepshead minnow was less than that of other estuarine species; e.g., in acute studies it was approximately ten times less sensitive to kepone than was *L. xanthurus* (*42*).

In chronic, life-cycle toxicity studies, *C. variegatus* has also shown its responsiveness and usefulness. For example, with kepone, detrimental effects were observed in *C. variegatus* at concentrations a thousandfold less that of previously reported 96-hour LC50 values (*43*). Results of chronic endrin toxicity tests, on the other hand, corroborated acute data for the chronic effects level of 0.31 μg/liter and acute 96-hour LC50 concentrations of 0.34–0.37 μg/liter (*36*). These data and others suggest that *C. variegatus* is an indicative and sensitive estuarine species.

The use of *F. grandis* as a toxicological test organism has not been as extensive as that of *C. variegatus;* however, several studies indicate that it may be a responsive, useful estuarine species. For example, Ernst and co-workers (*30*) noted pathological effects in embryos exposed to 25% concentrations of the water-soluble fraction of No. 2 fuel oil, a concentration unlikely to be found in the aqueous environment but potentially available in petroleum-laden sediments into which developing embryos may settle. Also, *F. grandis* has been used in investigations of toxicant effects on fin regeneration (*44*), of hexachlorobenzene and hexachlorobutadiene [20-hr LD50: 1,700 μg/kg body wt observed for the latter chemical (*45*)], and numerous studies involving the effects of salinity, temperature, photoperiod, and other parameters on a variety of serum constituents (*46–50*). Although these latter citations do not involve toxicological testing, they do illustrate an important consideration in the use of *F. grandis* in carcinogen assay when compared with smaller species such as *C. variegatus,* i.e., that of the adaptability of *F. grandis* to serological studies. The adult *C. variegatus* averages 48 mm standard length for males and 45 mm for females and is not an optimal test animal for blood serum studies. Pooled sample methods have been performed at our laboratory with some difficulty and with fairly large numbers of fish required (*51*). The small size of *Cyprinodon* and low volume of collectable blood make individual samples impractical. *F. grandis,* however, the standard length of which can range to 100 mm and greater, is large enough to permit blood chemistry studies of individuals, some of which are currently under consideration.

PREVALENCE OF IDIOPATHIC TUMORS IN *CYPRINODON* AND *FUNDULUS*

Examination of hundreds of specimens of *C. variegatus* from both feral and laboratory populations provides little evidence that the sheepshead minnow has a high natural

[4] Use of trade names in no way indicates endorsement by the U.S. Environmental Protection Agency.

TABLE 2.—*Idiopathic tumors reported in* F. heteroclitus, F. grandis, *and* C. variegatus[a]

Species	Tumor type	Reference
C. variegatus	Thyroid (adenoma)	(*52*)
	" "	(*53*)
	Odontogenic (ameloblastoma)	(*54*)
F. heteroclitus	Thyroid (adenoma)	(*52*)
	Integumentary (fibroma)	Yevich PP: Submitted for publication
	"	RTLA Spec. No. S0000204
	" (fibroepithelioma)	Paperna I: Submitted for publication
		RTLA Spec. No. S0000762
F. grandis	Possible stem cell (probable chromatophoroma)	Couch JA: Unpublished observation

[a] Data are from the Smithsonian Institution: Registry of Tumors in Lower Animals.

tumor prevalence. We have yet to find an invasive idiopathic neoplasm in *Cyprinodon*. A possible explanation for this low incidence is that fish, burdened with neoplasia, would be subject to a higher degree of predation than normal (due to decreased mobility, disorientation, etc.) and, therefore, would not be collected. The literature (table 2) also suggest tnat neoplastic development is rare in this species. Nigrelli (*52*) reported thyroid tumors in *C. variegatus* from the New York Aquarium that were characterized by increased follicular and afollicular elements. The tumors were classified as interacinous adenomas. Another aquarium-held sheepshead minnow with a similar tumor was reported by Lightner and Meineke (*53*); however, their tumor was classified as a benign adenoma rather than a malignant interacinous adenoma as indicated by Nigrelli (*52*). Thyroid enlargements were noted in *C. variegatus* held in aquaria at our laboratory; histological examinations by one of us (Couch) indicated thyroid hyperplasia. The only other tumor in this species we found mentioned in the literature (*54*) is an odontogenetic tumor (table 2).

Thyroid tumors or hyperplasia similar if not identical to those described in *C. variegatus* have also been reported (table 2) in *F. heteroclitus* from the New York Aquarium (*52*).

In addition, 3 specimens of *F. grandis* with suspect multicolored chromatophoromas composed of cells containing mosaics of different pigment organelles (fig. 9) have been studied at our laboratory by Couch. The fish were obtained from a mariculture facility on the Gulf Coast of Alabama, and were the older, larger specimens of over 6,000 fish examined. Detailed light microscopy (figs. 10, 11), electron microscopy, chromatographic, and cell culture studies were conducted on the tumors. The tumor cells possessed apparent pterinosomes as the dominant cytoplasmic organelle, but many cells also possessed possible premelanosomes, melanosomes, and possibly melanized reflecting platelets. The major pigments isolated and identified were pteridine pigments. Black pigments presumed to be melanin (fig. 10) were observed by light and electron microscopy. No carotenoid pigments or carotenoid vesicles were identified or detected. Couch proposed that the tumors may have arisen from neoplastic stem pigment cells or from anaplastic pigment cells. The tumors were invasive (figs. 10, 11) and replaced and isolated normal muscle tissues, but they were not metastatic. Chromatophoromas of 1 specimen, which eventually died in the aquarium, grew and increased in numbers. The cause of the neoplasm in *F. grandis* is unknown, but both environmental and genetic factors may be involved in its genesis.

CARCINOGEN-INDUCED LESIONS

We have worked with *C. variegatus* for 3 years to determine its potential as a carcinogen assay organism in long- and short-term exposure in critical life-stage tests with suspect and known carcinogens. Furthermore, we and B. J. Martin, of the University of Southern Mississippi, have examined *C. variegatus* for its responsiveness to at least 3 carcinogens in recirculating test systems, using both water and feeding as exposure routes. A brief report of the results, to date, of tumor induction tests with 1) trifluralin, a herbicide, 2) benzidine, and 3) aflatoxin B_1 follows. Each test was designed and performed in different modes and for different purposes; for the most part, environmentally realistic exposure concentrations (low parts per billion range) were tested.

Trifluralin

Embryos and 30-day old postfertilization fry were exposed to 1–5 μg trifluralin/liter for 19 months in a test system previously noted. Although no invasive neoplasms were found in these fish, several lesions were formed as a result of the chronic exposure (*38, 55, 56*). Most significant was a high prevalence of diffuse vertebral dysplasia, focal hyperostosis in the backbone, and apparent pituitary enlargement and histopathological changes. The vertebral dysplasia was characterized by the abnormal persistence of osteoblastic cells that continued to form cartilage and bone. Although Couch et al. (*38, 55, 56*) found this to be a persistent cellular phenomenon (stimulated by trifluralin), perhaps combined with a minor cellular proliferative one, the end result was the production of focal benign growths in the vertebrae. Pathobiologists and biologists working in fisheries should be aware of this type of lesion and avoid confusing it with neoplasia, particularly in fish from feral populations, but also in those exposed to carcinogens in the laboratory.

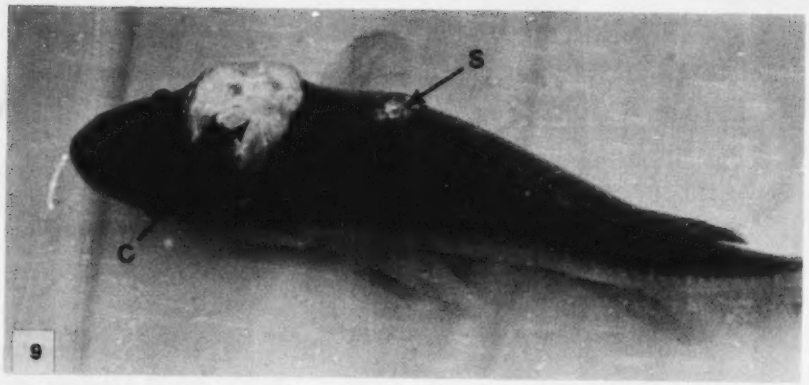

FIGURE 9.—Adult *F. grandis* with suspect chromatophoroma (C) protruding from dorsal surface of anterior trunk. Secondary tumor (S) protrudes posterior to large tumor.

FIGURE 10.—Tumor cells (T) invade muscle bundles (M), gradually surrounding and isolating them. Black pigments (P), presumed to be melanin, are deposited throughout the tumor site. Chromatographic analyses revealed pteridine pigment in these cells. H & E. × 125

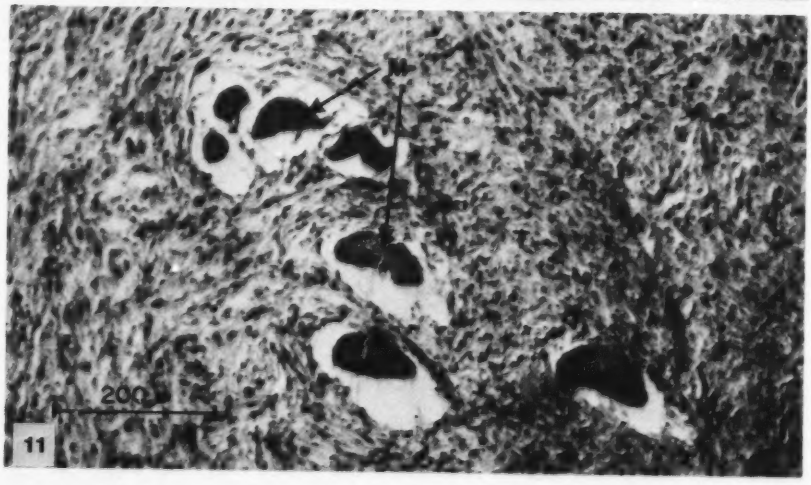

FIGURE 11.—Muscle bundles (M) are completely isolated by invading tumor cells (T) and appear partially lysed. H & E. × 125

Benzidine

Apparent proliferative liver lesions were produced in *C. variegatus* exposed to 1 ppm benzidine dihydrochloride in recirculating systems (Martin BJ: Personal communication). Exposures were pulsed at 12-day intervals for 166 days and longer, at which time several fish became moribund. Histological examination revealed lesions involving tubular proliferation of either bile ductules or vascular tubules in the livers. Work continues on identification; however, preliminary results suggest that these lesions are hemangioendotheliomas, cholangiocarcinomas, bile duct adenomas, or merely adenofibrosis.

Aflatoxin B_1

To test the responsiveness of *C. variegatus* to an overt carcinogen, we exposed embryos and larvae (critical life stages) to aflatoxin B_1. This compound produces a high incidence of hepatocellular carcinomas in rainbow trout exposed as embryos to 500 ppb aflatoxin B_1 (*39*). The 96-hour-old embryos and 120-hour-old, newly hatched fry were exposed in a glove box under static conditions for 1 hour to 200 μg aflatoxin B_1/liter with dimethyl sulfoxide as a carrier. Embryos at this age were chosen because, at this stage, liver tissue, the apparent target tissue of aflatoxin B_1, is differentiated and well developed (figs. 5, 6); however, the embryos were encased in the chorion and penetration of the compound presented a possible problem. Therefore, we also exposed newly hatched fry (120 hr old) to circumvent the penetration problem. After exposure, the animals were transferred to static 200-liter holding aquaria equipped with under-substrate filters and maintained for 6 weeks. Then they were transferred to and are still being maintained in 38-liter aquaria with flowing water. Fish have been taken at 1 month, 3 months, and 6 months postexposure for histological examination. The most significant finding to date is a high incidence of mitotic figures in the hepatocytes of the exposed fish from the 1-month sample. Hepatic cell mitotic activity (fig. 12) nine times that of controls was noted in exposed fish. Later samples failed to reveal any significant lesions, nor was unusual mitotic activity noted. The fish continue to be maintained and additional sampling is scheduled for 12 months.

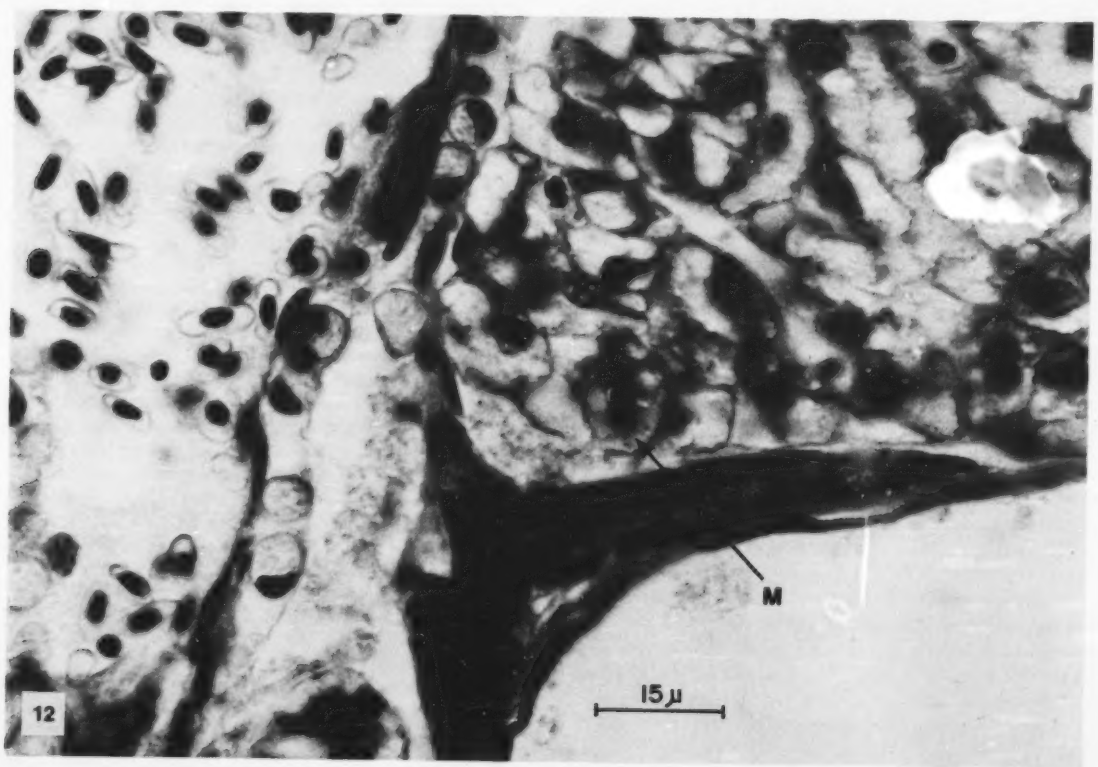

FIGURE 12.—Liver section from 1-mo-old *C. variegatus* exposed as 120-hr fry to 200 μg aflatoxin B_1/liter for 1 hr. *Note* mitotic figure (M). Mitotic activity nine times greater than control was observed in exposed specimens. H & E. × 1,325

ADVANTAGES AND PROBLEMS WITH *CYPRINODON* AND *FUNDULUS* AS CARCINOGEN TEST SPECIES

C. variegatus and *F. grandis* possess many assets that make them desirable test organisms. Although certain problems must be considered when they are used in cancer research, the advantages presented by *C. variegatus* and *F. grandis* far outweigh the possible problems encountered with their use; the potential of these species should be fully explored.

In summary, many advantages are exhibited by both species. These include: 1) wide geographic distribution; 2) abundant, ecologically significant estuarine species; 3) transportability; 4) easy culture and maintenance; 5) environmental adaptability (e.g., salinity, temperature); 6) typical teleostean anatomy and histology and thus adaptability to routine and special histological techniques; 7) easily fulfilled nutritional requirements; and 8) documented history of morphological and physiological responsiveness to toxic chemicals (especially with *C. variegatus*). Moreover, both species are euryhaline, but, in estuarine and marine water, they drink the seawater and thus expose the entire alimentary canal to a water-borne carcinogenic agent.

Each species has, in addition, its specific advantages. The *C. variegatus* offers 1) a small organism that required limited holding space, 2) short embryological development period, 3) short generation time, and 4) a large toxicological data base. *Fundulus,* on the other hand, offers 1) a larger organism that is more easily adaptable to certain special procedures (e.g., serum or injection studies), 2) extended developmental and generation periods, and 3) larger organ systems for use in biochemical studies.

Problems encountered with these species are, for the most part, also experienced with most other test animals. The fishes are susceptible to some infectious and noninfectious disease agents often encountered in the natural history and culture of teleost species. For example, monogenetic trematodes (*Gyrodactylus* spp.) are sometimes a problem with *C. variegatus. Eimeria funduli,* a pathogenic coccidial protozoon, infects *F. grandis* and may greatly affect growth (*57*). However, the disease problem with these species is not exceptional. We have encountered no major problems with disease in several years of study, and with control of extreme environmental fluctuations, these species appear less susceptible to culture-related diseases than many others.

An additional potential problem that is widespread with many fish species is the penetrability of the chorionic membrane by test compounds. To circumvent this problem, we are studying the usefulness of penetrants, such as dimethyl sulfoxide. Additionally, dechorionation methods (*58, 59*) may be feasible. Moreno (*58*), using *C. variegatus,* describes a method involving penetration of the chorion with the sharpened end of a pair of forceps and gently rolling the egg under the blade of a scalpel until the chorion is excised. This is essentially a shelling out process and can be performed with minimal damage to the embryo.

Of final and central importance is an apparent low sensitivity to suspect carcinogenic agents noted in our studies. The spectrum of agents tested thus far, however, has been limited, and we have purposely used environmentally realistic, low concentrations. Though neoplastic responses have been dubious, some suspect proliferative lesions have been induced with specific agents (e.g., benzidine induction of proliferative tubular elements in the liver of *C. variegatus*). More intensive and diverse experimentation with a broader spectrum and higher concentrations of suspect carcinogenic agents for the evaluation of the responsiveness of both species is warranted.

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DISCUSSION

J. D. Hendricks: What were the concentrations and times of exposure of *Cyprinodon* embryos to aflatoxin B_1?

J. A. Couch: We used 200 μg/liter for 1 hour for the 96- and 120-hour embryos.

A. Woodhead: What are the frequencies and significance of the aging pigments in the livers that you showed?

Couch: We find that livers from fish that are diseased, stressed, or exposed to toxic chemicals may have larger amounts of the hemosiderin or melanin-macrophage centers than do normal livers from healthy fish. Even some apparently healthy fish from nature have relatively large amounts of these pigments. They seem to be related to injury when they occur in large amounts.

G. Streisinger: Is it possible to collect ova and especially sperm without killing the animal, e.g., using capillary tubes to collect milt?

L. A. Courtney: Collection of ova from ripe females can and has been done with *Cyprinodon variegatus*. Dissection is usually used for two reasons: When human chorionic gonadotropin injections are used, the possibility of subsequent infection exists, and therefore the ova are not manually stripped. We use dissection instead. When human chorionic gonadotropin is not given, manual stripping works quite well, and females remain healthy and continue to produce. Collection of sperm from males by capillary tubing has not been done in our laboratory; however, I believe it could be done easily with either species (*Cyprinodon* or *Fundulus*).